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Plasmid pCS1966, a New Selection/Counterselection Tool for Lactic Acid Bacterium Strain Construction Based on the *oroP* Gene, Encoding an Orotate Transporter from *Lactococcus lactis*▽

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In this paper we describe the new selection/counterselection vector pCS1966, which is suitable for both sequence-specific integration based on homologous recombination and integration in a bacteriophage attachment site. This plasmid harbors *oroP*, which encodes a dedicated orotate transporter, and can replicate only in *Escherichia coli*. Selection for integration is performed primarily by resistance to erythromycin; alternatively, the ability to utilize orotate as a pyrimidine source in a pyrimidine auxotrophic mutant could be utilized. Besides allowing the cell to utilize orotate, the transporter renders the cell sensitive to 5-fluoroorotate. This sensitivity is used to select for loss of the plasmid. When expressed from its own promoter, *oroP* was toxic to *E. coli*, whereas in *Lactococcus lactis* the level of expression of *oroP* from a chromosomal copy was too low to confer 5-fluoroorotate sensitivity. In order to obtain a plasmid that confers 5-fluoroorotate sensitivity when it is integrated into the chromosome of *L. lactis* and at the same time can be stably maintained in *E. coli*, the expression of the *oroP* gene was controlled from a synthetic promoter conferring these traits. To demonstrate its use, a number of *L. lactis* strains expressing triosephosphate isomerase (*tpiA*) at different levels were constructed.

Construction of tailor-made strains is dependent on efficient genetic methods, and in order to obtain genetically stable strains, chromosomal integration is often desirable. This calls for techniques that allow efficient selection of both chromosomal integration and excision. For many years, plasmids unable to replicate but expressing antibiotic resistance in *Lactococcus lactis* have been used to obtain strains in which the plasmid has been integrated into the chromosome. Whereas the isolation of integrants was straightforward, strains that had lost the plasmid were not as easy to obtain. A genetic tool based on a plasmid whose replication is impaired at high temperatures has been used extensively to obtain chromosomal insertions and deletions in *L. lactis* (2). While the integration is dependent on selection for antibiotic resistance at a nonpermissive temperature, the excision step relies on lowering the temperature to the permissive temperature, taking advantage of the growth inhibition resulting from initiation of rolling circle replication from the plasmid origin located on the chromosome (2). Besides inducing genetic instability, a disadvantage of this system is that the nonpermissive temperature is 37°C, which is at the limit for growth of a number of lactococcal strains. A gene involved in nucleotide metabolism has been demonstrated to work as a counterselection marker; loss of the *upp* gene encoding uracil phosphoribosyltransferase results in resistance to 5-fluorouracil (3, 14, 15). The main drawbacks of using *upp* are that this gene is found in almost every organism and that 5-fluorouracil may be toxic even in a *upp* mutant (14, 15).

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L. lactis synthesizes pyrimidines de novo, but it is also able to metabolize various exogenous pyrimidine nucleosides and nucleobases (12). The de novo pyrimidine pathway consists of six steps leading to the formation of UMP (10). Orotate is the fourth intermediate in the pathway, and it has been shown that this compound cannot be utilized by most *L. lactis* strains as a pyrimidine source because the strains lack a transporter (7, 10). After screening a number of *L. lactis* strains, we isolated one strain that was able to take up orotate due to the presence of plasmid pDBORO encoding a dedicated orotate transporter. The *oroP* gene identified on the plasmid was shown to confer the ability to utilize orotate as a sole pyrimidine source in an auxotrophic mutant, and the presence of this gene results in sensitivity to the toxic analogue 5-fluoroorotate (7). These findings suggested that the *oroP* gene can be exploited as an efficient selection/counterselection marker. Moreover, it was found that *oroP* was functionally expressed in both *Escherichia coli* and *Bacillus subtilis*, suggesting that it can be utilized as a selection/counterselection marker in wide range of bacteria (7, 13). When the *oroP* allele was introduced into the chromosome of *L. lactis*, no significant 5-fluoroorotate sensitivity was acquired, whereas when the same *oroP* allele was localized on the chromosome of *B. subtilis*, the cells became sensitive to 5-fluoroorotate, thus making the original allele suitable for genetic recombination on the *B. subtilis* chromosome (7). In this paper we describe pCS1966, a new vector harboring the *oroP* gene suitable for obtaining chromosomal recombination in *L. lactis* using selection in the presence of erythromycin or orotate and counterselection in the presence of 5-fluoroorotate.

Construction of a selection/counterselection tool for *L. lactis*. Like most integration vectors for bacteria other than *E. coli*, pLB85 is based on a plasmid that is able to replicate only in *E.*

TABLE 1. Primers used in this study

Primer	Sequence
AttP-F	5′-CGTAGCCGTGCGGCCGCCAACAACCTTGTATCAAAGG-3′
AttP-R	5′-TGCTGGGAGCTCCCTTCTATGCATGAGATAAC-3′
105	5′-CGGTCGACACGCTGTTCCATATTACTAG-3′
106	5′-ACGACTAGTGGATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNTGRTATAAT NNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGTATATTTACCTAGCTTTTGC-3′
CPOro-F	5′-GGAAGGATCCCCCATAGTTCATCAGTTATC-3′
CPOro-R	5′-ATACGACTCACTATAGGGC-3′
TpiUp-F	5′-GGTACTCGAGCAGAAAGTTAGGTTATGATGG-3′
Tpi-R1	5′-GGAAGGATCCTCATCATTAATAAATTTCAATCC-3′
T7	5′-TAATACGACTCACTATAGGG-3′
Tpi-R2	5′-TGGTATCTAGAGTTGAACGAACAACACCAC-3′

coli (4). During attempts to clone the *oroP* gene it was observed that a derivative of the *L. lactis* integration vector pLB85 in which the *oroP* gene was inserted could not be established in *E. coli*. This was probably due to expression of the *oroP* gene, which encodes a membrane protein, that was too high (data not shown). On the other hand, when this plasmid was introduced into the chromosome of *L. lactis*, no 5-fluoroorotate sensitivity was acquired (data not shown), whereas when the same *oroP* allele was localized on a plasmid, the cell became sensitive to 5-fluoroorotate (7). This indicated that the *oroP* promoter is relatively strong in *E. coli* and relatively weak in *L. lactis*, which is actually opposite the properties required for a selection/counterselection tool for *L. lactis*. We therefore decided to see whether modulating the strength of the *oroP* promoter using a synthetic promoter library resulted in a derivative with reduced expression in *E. coli* and, simultaneously, increased expression in *L. lactis*. In order to meet the requirement for integration of the vector alone in the chromosome of *L. lactis*, plasmid pCS1861 was created. The bacteriophage TP901-1 attachment site *attP* (6) was amplified by PCR from pLB85 using primers AttP-F and AttP-R and, after digestion with NotI and SacI, fused to plasmid pRC1 (11) linearized with the same enzymes. After transformation into *E. coli* ABLE-C (Stratagene), which was *E. coli* C *lac*($LacZ^-$) [Kan^r $McrA^-$ $McrCB^-$ $McrF^-$ Mrr^- HsdR (r_k^- m_k^-)] [F′ *proAB* $lacI^qZ\Delta M15$ Tn*10*(Tet^r)], the resulting plasmid, pCS1861, was selected on LB medium containing 150 μg/ml erythromycin. We routinely use ABLE-C, which lowers the copy number of ColE1 derivatives, to reduce cloning problems due to toxicity of cloned fragments in *E. coli*. A PCR fragment in which a promoter library expressing *oroP* was generated by PCR using primers 105 and 106 (Table 1). A mixture of PCR fragments was digested with SpeI and SalI and ligated into pCS1861. Several hundred *E. coli* ABLE-C transformants were obtained on LB medium supplemented with 150 μg/ml erythromycin. The transformants were washed off the plates and used to inoculate liquid LB medium supplemented with erythromycin. The mixed culture was allowed to grow for at least 10 generations in order to enrich for strains that grew well. Plasmid DNA was extracted and used for electroporation (8) in order to transform the *L. lactis* pyrimidine-requiring derivative ED79.1175 (*pyrDa pyrDb*) (7). To ensure that the plasmid integrated at the bacteriophage TP901-1 attachment site, ED79.1175 was transformed in advance with plasmid pLB65 (4), which encodes the integrase from TP901-1 (6). In order to obtain an integrant that harbored an allele of *oroP* that was sufficiently expressed in *L. lactis*, the transformants were plated on chemically defined SA medium (9) supplemented with 1% glucose and with 20 μg/ml orotate as a sole pyrimidine source. A number of transformants were obtained, and their dependence on orotate for growth was confirmed by restreaking on the defined medium in the absence and presence of orotate. The plasmids from the integrated strains were recreated by PCR amplifying a fragment containing the synthetic promoter and the *oroP* gene using chromosomal DNA isolated from the orotate-positive strains as the template and primers CPOro-F and CPOro-R (Table 1). After digestion with SacI, the fragments were inserted into pCS1861 by ligation and transformed into *E. coli* ABLE-C. Plasmid DNA was purified and used to transform *L. lactis* MG1363 harboring pLB65. Transformants were selected on M17 plates supplemented with 1% glucose and 5 μg/ml erythromycin. The strains were screened on SA medium supplemented with 1% glucose and 10 μg/ml 5-fluoroorotate. Among the transformants found to be sensitive to 5-fluoroorotate, plasmid pCS1966 (Fig. 1) was chosen for further study. The DNA upstream of *oroP* in pCS1966 was sequenced, and the putative promoter was identified (Fig. 1). A 4-bp deletion between the −35 and −10 boxes in the promoter sequence was observed. Normally, the space between the two elements is 17 bp long, and therefore the consensus −35 se-

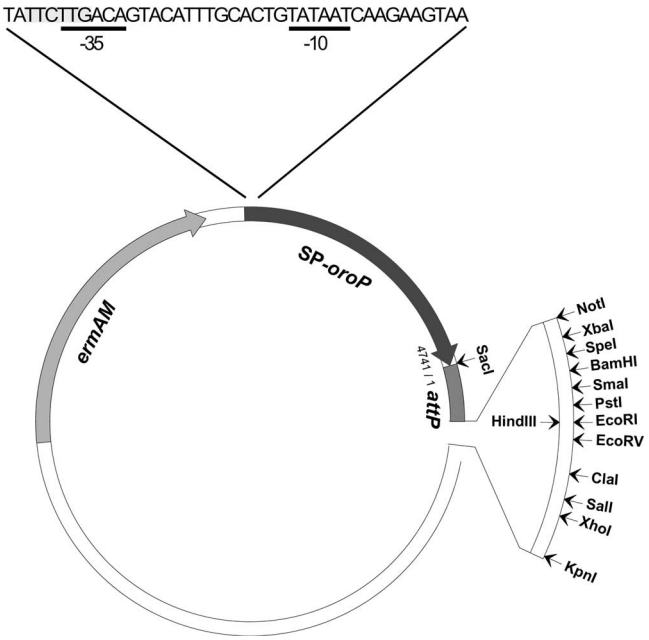

FIG. 1. Selection/counterselection vector pCS1966. The *oroP* gene encodes the orotate transporter expressed from the synthetic promoter obtained by selection (SP-*oroP*). The *ermAM* gene confers erythromycin resistance. The *attP* gene is the attachment site from bacteriophage TP901-1. Unique restriction enzyme sites are indicated. The sequence of the promoter driving the expression of *oroP* is shown at the top. The designed consensus −35 and −10 sequences are underlined, and the alternative −35 sequence is indicated by shading.

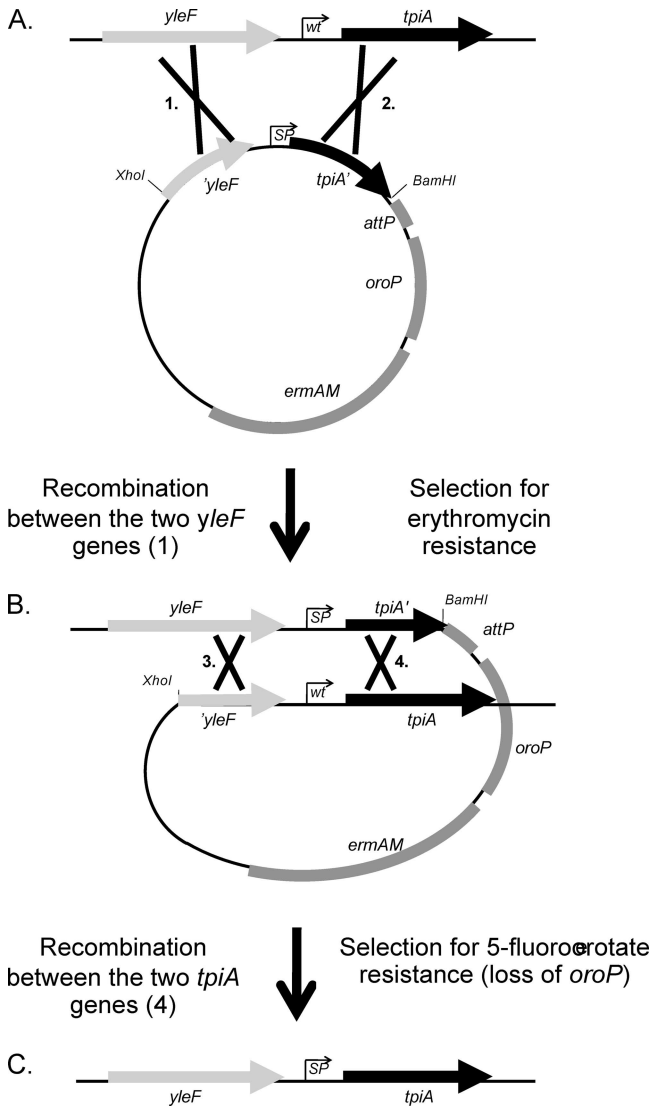

FIG. 2. Scheme for exploitation of the *oroP*-based selection/counterselection vector pCS1966 for construction of strains expressing the *tpiA* gene encoding triosephosphate isomerase from synthetic promoters. (A) A derivative of pCS1966 carrying the *ylfE-tpiA* region from *L. lactis* IL-1403 in which the normal promoter has been replaced by a synthetic promoter is subject to homologous recombination in IL-1403. The recombination takes place at either *yleF* (recombination 1) or *tpiA* (recombination 2). The integration is selected by erythromycin resistance. (B) Outcome of the recombination involving interaction between the *yleF* genes (recombination 1). A second recombination event involving either *yleF* (recombination 3) or *tpiA* (recombination 4) results in excision of a plasmid. Only strains with plasmids excised are viable in the presence of 5-fluoroorotate. When the recombination involves *yleF* (recombination 3), the integration is reversed, resulting in regeneration of the wild type. (C) Result of excision by a recombination event at *tpiA* (recombination 4). The wild-type and synthetic promoters are indicated by arrows and designated *wt* and *SP*, respectively. *ermAM* confers erythromycin resistance, *attP* is the attachment site from bacteriophage TP901-1, and *oroP* encodes the orotate transporter from *L. lactis*. The XhoI and BamHI restriction enzyme sites are indicated.

quence (TTGACA) is probably not involved in primary promoter recognition; instead, the highly degenerate TTCTTG sequence may serve as the initial RNA polymerase binding site (Fig. 1). This could account for the poor expression in *E. coli*. For *L. lactis* the fact that this promoter is stronger than the wild-type promoter could indicate that the original *oroP* promoter is extremely weak.

Use of the selection/counterselection tool to construct genetically stable *tpiA* derivatives. To test the usefulness of pCS1966, it was used to construct a number of *L. lactis* subsp. *lactis* IL-1403 (5) strains with synthetic promoters upstream of the *tpiA* gene encoding triosephosphate isomerase. Plasmid pCS2055 was constructed by inserting the *tpiA* upstream region generated by PCR with primers TpiUp-F and Tpi-R1 (Table 1) between the XhoI and BamHI sites in the *oroP* vector pCS1966. Chromosomal DNA was isolated from four strains containing synthetic promoter plasmids integrated into the *tpiA* locus and expressing *tpiA* at different levels (C. Solem, unpublished), and a PCR fragment was generated using primers T7 and Tpi-R2 (Table 1). The resulting PCR fragments were digested with BamHI and XbaI and cloned in pCS2055 containing the *tpiA* upstream region (Fig. 2). The four plasmids were amplified in *E. coli* ABLE-C, purified, and used to transform *L. lactis* IL-1403. Strains in which the plasmids were integrated into the chromosome were obtained on M17 plates supplemented with glucose and erythromycin as the plasmids did not replicate in *L. lactis*. Integration occurred by homologous recombination either at the *tpiA* open reading frame or at the upstream sequence (Fig. 2). The transformants were purified and plated on SA glucose plates supplemented with 5-fluoroorotate (10 μg/ml), thereby selecting for strains in which the plasmid had been lost by homologous recombination. The recombination may have taken place either at the *tpiA* open reading frame or at the upstream sequence (Fig. 2). In both cases the plasmid was lost, leaving either the wild-type or the synthetic promoter upstream of the *tpiA* open reading frame. Among the 5-fluoroorotate-resistant strains, the clones expressing *tpiA* from a synthetic promoter were identified by PCR. Finally, to verify that the different strains expressed *tpiA* at different levels, the triosephosphate isomerase levels were assayed using crude extracts made from the strains growing exponentially in SA medium. The crude extracts were prepared as described by Andersen et al. (1), except that the cells were disrupted with glass beads. As shown in Table 2, the enzymatic activities did vary, as expected.

We concluded that the pCS1966 vector described in this paper can be used as an efficient tool for strain construction. As the *oroP* gene is functionally expressed in different bacteria,

TABLE 2. Relative triosephosphate isomerase activities in derivatives of *L. lactis* IL-1403

Strain	Relative triosephosphate isomerase activity[a]
IL-1403	1
CS2116	0.24
CS2110	0.33
CS2127	0.83
CS2121	0.91

[a] Triosephosphate isomerase activity in crude extracts was determined by spectroscopic determination of the consumption of NADH at 340 nm. The reaction mixture contained 125 mM triethanolamine buffer (pH 7.2), 0.3 mM NADH, glycerol-3-phosphate dehydrogenase (1 U), and 6 mM glyceraldehyde-3-phosphate. All measured enzyme activities were related to the optical density at 280 nm of the extract. The specific activity in IL-1403 was defined as 1.

including *L. lactis*, *E. coli*, and *B. subtilis*, the counterselection procedure using 5-fluoroorotate described in this work can potentially be exploited in a large number of organisms. However, the strain must be devoid of an orotate transporter. If an orotate transporter is present, a derivative lacking this ability can easily be obtained by selection on plates containing 5-fluoroorotate.

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